I. Synthesis and characterization of a FXIII inhibitor

A dodecapeptide inhibitor of activated factor XIII (FXIIIa) was synthesized that mimics the sequence of the N-terminal part of α_2-plasmin inhibitor, an excellent substrate of FXIIIa. In the sequence the substrate glutamine at position 2 was replaced by homocysteine: N-hCys-E-Q-V-S-P-L-T-L-L-K. The synthesis was upgraded and the peptide was purified by HPLC. The expected molecular mass was verified by MALDI-TOF. The peptide showed a dose-dependent inhibition of activated FXIII (FXIIIa) with an IC50 of 0.138 mM. The irreversible covalent binding between the hCys petide and FXIIIa was demonstrated by MALDI-TOF technique. The molecular mass of FXIIIa was 79 kD, while that of the inhibited FXIII was 80.3 kD showing that the hyCys peptide (molecular mass: 1.3 kD) was incorporated into FXIIIa. Comparison of the tryptic digest of FXIIIa and hCys-FXIIIa also demonstrated the covalent attachment of inhibitory peptide to a peptide containing the active site cysteine in FXIIIa. A further proof was that the inhibition was abrogated in the presence of the reducing agent ditiotreitol. It was also tested how the hCys peptide influences the physiological functions of FXIIIa. It blocked the cross-linking of fibrin γ-chain dimerization and fibrin α-chain polymerization. It also interfered with with the cross-linking of α2 plasmin inhibitor to fibrin. Due to the relatively low affinity of the hCys peptide to FXIIIa it cannot be considered as a potential therapeutically utilizable FXIIIa inhibitor, however it may provide a lead for designing more powerful inhibitors.

In addition to the original plan we also advanced into a new direction of depressing FXIII activity in the plasma. In the tetrameric plasmatic form of FXIII (FXIII-A_2B_2) the two potentially active FXIII-A subunits are associated with two inhibitory FXIII-B subunits. We showed that this is a tight association with a Kd in the range of 10^{-10} M. FXIII-B is needed to prolong the half-life of FXIII-A from a few hours to 8-14 days in the circulation. Thus, the inhibition of complex formation could regulate plasma FXIII level. We produced an anti-FXIII-B monoclonal antibody, which reacts only with the free FXIII-B and prevents the complex formation. We were able to show that the binding epitope for antibody is on the combined 1+2 sushi domain of FXIII-B synthesized in insect cells and in further experiments it was revealed and the sequence of the peptide responsible for the antibody binding was YGCASGYK (position 96-103) in the 2nd sushi domain. The results have been published (Katona et al. Blood 2014; 123: 1757-63). Pursuing this line we synthesized the YGCASGYK peptide and used in surface plasmon resonance experiments to test its effect on FXIII-A2-FXIII-B interaction. To our disappointment, the peptide had only a weak effect on the interaction. This finding suggested that the antibody does not bind to a FXIII-B epitope involved in the interaction with FXIII-A2, but it binds close to such epitope and exerts its effect by steric inhibition. To find the FXIII-A binding epitope(s) on FXIII-B we attempted to synthesize the first and second sushi domains of FXIII chemically in collaboration with the Department of Medical Chemistry, University of Szeged. The synthesis was successful and experiments with the individual sushi domains are in progress. A further line we followed in exploring the interaction between the two FXIII subunits was investigating patients with anti-FXIII-A antibodies (Muszbek and Katona Semin Thromb Hemost 2016;42:429-39, Muszbek et al. J. Thromb Haemost 2018;16:822-32) and trying to find antibodies that prevent the interaction between the two subunits. An international collaboration has been initiated and we were already able to collect 4 patients with anti-FXIII-A autoantibodies. However, their analysis will be the topic of a separate project.

II. FXIII and atherosclerosis

IIa) Detection of factor XIII (FXIII) within the atherosclerotic plaque and in arterial thrombi

Artery specimens with atheroscleorotic plaques obtained by surgery and from post-mortem material were investigated using immunohistochemical techniques. Introducing double immunofluorescent staining we were able to confirm and extend our finding based on simple immunoperoxidase staining. Within the plaque, where there was intra-plaque hemorrhage, extracellular FXIII-A was co-localized

with fibrin. Platelets present in the hemorrhagic part of the plaque showed bright FXIII-A fluorescence. Outside the hemorrhage, FXIII-A was detected in cells positive for monocyte, macrophage markers. The results also suggested that macrophages transformed into foam cells retain their FXIII content. To establish the role of FXIII in foam cell formation, if any, we need to explore the behavior of monocytes from FXIII deficient patients which were diagnosed and characterized according to internationally accepted guidelines worked out in our laboratory (Muszbek and Katona Semin Thromb Hemost 2016;42:429-39). As inherited FXIII-A deficiency is very rare, in Hungary we have only 2 deficient adults, we organized an international collaboration, which includes 6 patients from Finland and 2 patients from Switzerland. The ethical approvals for another study (see the cornea section) have been received, but we are still waiting the approvals for the monocyte study.

We also started investigations on the expression of FXIII in arterial thrombi from peripheral vessels and from coronaries removed by aspiration thrombectomy after the onset of pain. 24 coronary thrombi were investigated and they were divided according to the period the elapsed between the onset of pain and thrombectomy (<12 hours, 12-24 hours and >24 hours). In "fresh" thrombi FXIII-A was mainly located in platelets and extracellularly it was always associated to fibrin. Coronary thrombi obtained after 24 hours demonstrated a layered structure very likely due to inside-out fibrinolysis. An interesting finding was the close association of elastase positive granulocytes to fibrin (Figure 1) that suggests the importance of these cells in the fibrinolytic process. We also revealed neutrophil extracellular traps (NETs) in the thrombus. In separate experiments we demonstrated that protein crosslinking by transglutaminases (TGs) stabilizes NETs. Among the transglutaminases TG1 seems to play a major role. Isolated and PMA-activated neutrophils of a FXIII-A-deficient patient were able to form a functional NET structure suggesting that FXIII-A does not contribute to NET formation (Csomós et al. Cell Death Dis 2016; 7: e2332). However, in the hemorrhagic atherosclerotic plaque plasma FXIII might have direct contact with NET proteins and might be involved in its stabilization.

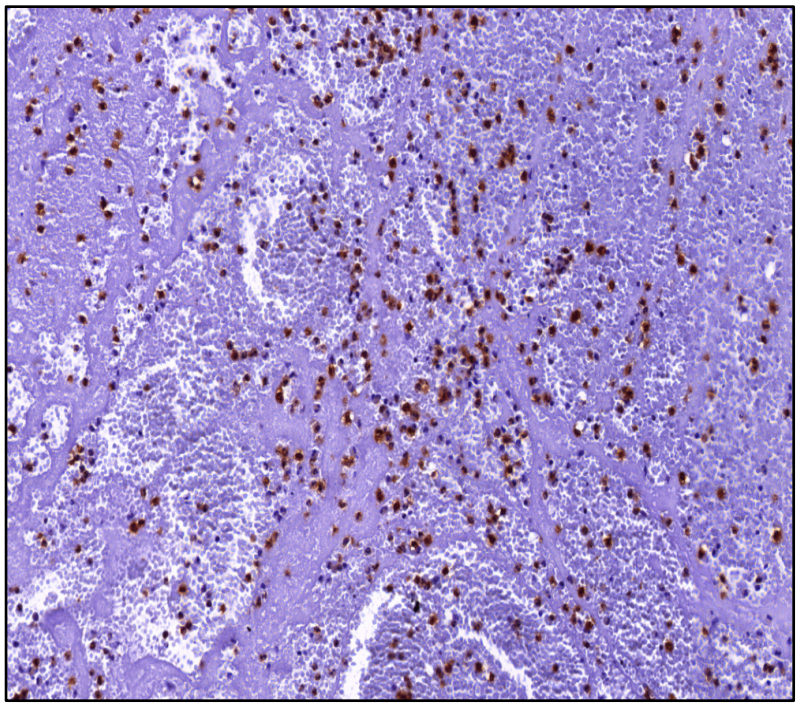

Figure 1. Elastase positive granulocytes in close proximity of fibrin strands.

IIb) The expression of FXIII in cultured vascular smooth muscle cells (VSMCs) and its relation to matrix formation and calcification.

This part of the planned study was based on earlier reports demonstrating that osteoblasts and osteocytes express FXIII-A, which contributes to matrix formation and mineralization in the bone. It was assumed that human aortic smooth muscle cell undergoing osteoblastic transformation also express FXIII-A. Unfortunately, the basic finding, the expression of FXIII-A in osteoblasts and osteocytes published by a couple of groups could not be confirmed and similar results were obtained by a group at the University of Leeds. It turned out that the anti-FXIII-A antibody purchased from the company Abcam by several authors cross-reacts with another protein. Using our monoclonal and monospecific polyclonal anti-FXIII-A antibodies we were not able to detect FXIII-A in human aortic smooth muscle cells (HAoSMC) undergoing osteoblastic transformation and involved in mineralization in response to elevated levels of inorganic phosphate. Thus we had to abandon this part of the project.

IIc) The effect of FXIII on vascular smooth muscle cells (VSMCs) 2014

The effect of non-activated and activated FXIII was tested on the proliferation and migration of of human aorta derived smooth muscle cells (HAoSMC). As the 0.2% fetal bovine serum used for culturing the cells contained a minute amount of bovine plasma FXIII, the medium was immunodepleted to remove even traces of FXIII. Non-activated FXIII was without effect on the proliferation of the cells, as tested by the EZ4U assay (Biomedica). Next we tested the effect of FXIII activated by thrombin and Ca^{2+}. As thrombin by itself increased cell proliferation, following FXIII activation thrombin had to be inhibited by recombinant hirudin. FXIIIa in the range of 2.5-20 μg/mL dose-dependently increased the proliferation of HAoSMCs. 15 μg/mL FXIIIa increased the extent of proliferation by 40%. It is to be noted that the effective FXIIIa concentration is comparable to that present in human plasma. Experiments with another proliferation assay (CCK-8) gave similar results.

In the next series of experiments the effect of FXIIIa on in vitro wound healing assay was tested. This assay measured the combined effect on cell proliferation and migration. Using CytoSelect 24-Well Wound Healing Assay the process of wound closure was video monitored by Juli Stage Real Time Cell History Recorder. Activated recombinant cFXIII (rFXIIIa), but not the non-activated form, accelerated the wound closure in a concentration dependent manner. The time to reach 30% and 80% confluence was decreased to less than 1/7 and 1/3 by the addition of 20 μg/mL cFXIIIa. A further novel finding was that FXIIIa induced increased collagen secretion by HAoSMCs into the extracellular matrix.

To explore the biochemical mechanism the effect of FXIIIa on thrombospondin-1 level in the medium and in the cells were also investigated. A highly significant (67%) decrease of thrombospondin-1 (Tsp-1) concentration in the medium and a 2.5-fold increase within the cells were observed (Figure 2).

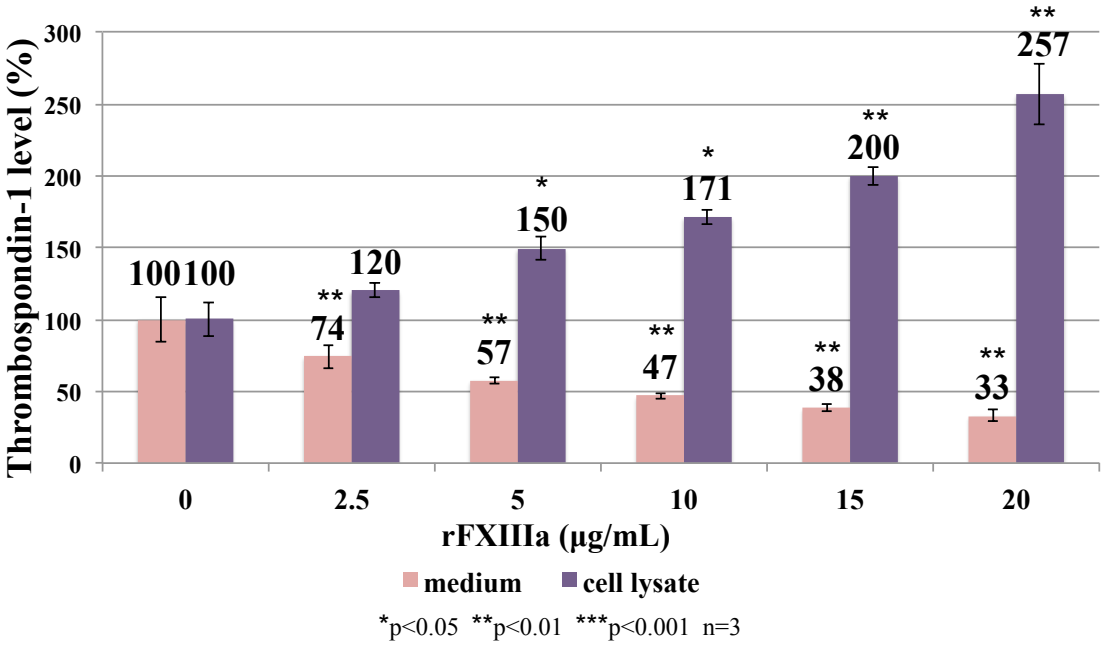

Figure 2. The effect of FXIIIa on thrombospondin-1 concentration in the medium and in the lysate of HAoSMC.

However, neither Tsp-1 nor c-Jun and Egr-1 mRNA levels changed significantly. This finding suggests that FXIIIa did not influence the synthesis of Tsp-1. It was shown by immunomorphological investigations that FXIIIa induced the formation of cell-associated precipitates, which did not contain Ca, but were intensively labeled for Tsp-1. The results suggest that the decrease of Tsp-1 in the medium is due to the formation of granules containing cell-associated Tsp-1.

The scientific material concerning HAoSMC was presented at the following international congresses/symposia:
2nd International Factor XIII Workshop September 25-October 2, 2016, Hévíz Hungary (Bogáti et al. Abstract No.: T5/4 "Factor XIII and vascular smooth muscle cells");
Transglutaminases in Medicine, August 3-6, 2017 Debrecen, Hungary (Muszbek L invited lecture "Coagulation factor XIII: a protransglutaminase with functions outside the coagulation cascade").
Transglutaminases in Human Disease Processes, Gordon Research Seminar, le Diablerets, France June 16-17 2018, (Bogáti et al. among the best 8 selected oral presentations: "The effect of factor XIII on the proliferation and migration of vascular smooth muscle cells").
International Society on Thrombosis and Haemostasis, SSC July 18-21, 2018 Dublin, Ireland (Muszbek L invited lecture "The effect of activated FXIII on human vascular smooth muscle cells").

We asked for extension of the project to complete the study and to reach the stage when the results can be submitted to a first class scientific journal. The manuscript is now in preparation.

III. Cellular FXIII in the cornea and its involvement in corneal wound healing 2014
a) FXIII-A in the cornea

It was demonstrated by immunofluorescent investigations that cells in human corneal stroma express the cellular form (cFXIII; FXIII-A_2) of coagulation FXIII, while neither epithelial nor endothelial cells were labeled by anti-FXIII-A antibody. No cell showed positive staining for FXIII-B. As demonstrated by laser scanning microscopy, FXIII-A+ cells showed co-staining for CD34, however, a significant number of CD34+ cells were negative for FXIII-A. While CD34+ cells were evenly

distributed throughout the stroma, FXIII-A+ keratocytes were unevenly distributed; they were abundant in the subepithelial tertile of stroma, while they were sparse in the subendothelial tertile (Figure 3). The existence of FXIII-A in the cornea was confirmed by Western blotting. 2.86 ng FXIII-A/mg corneal protein was measured by quantitative densitometry. The synthesis of FXIII-A by keratocytes was verified by measuring FXIII-A mRNA.

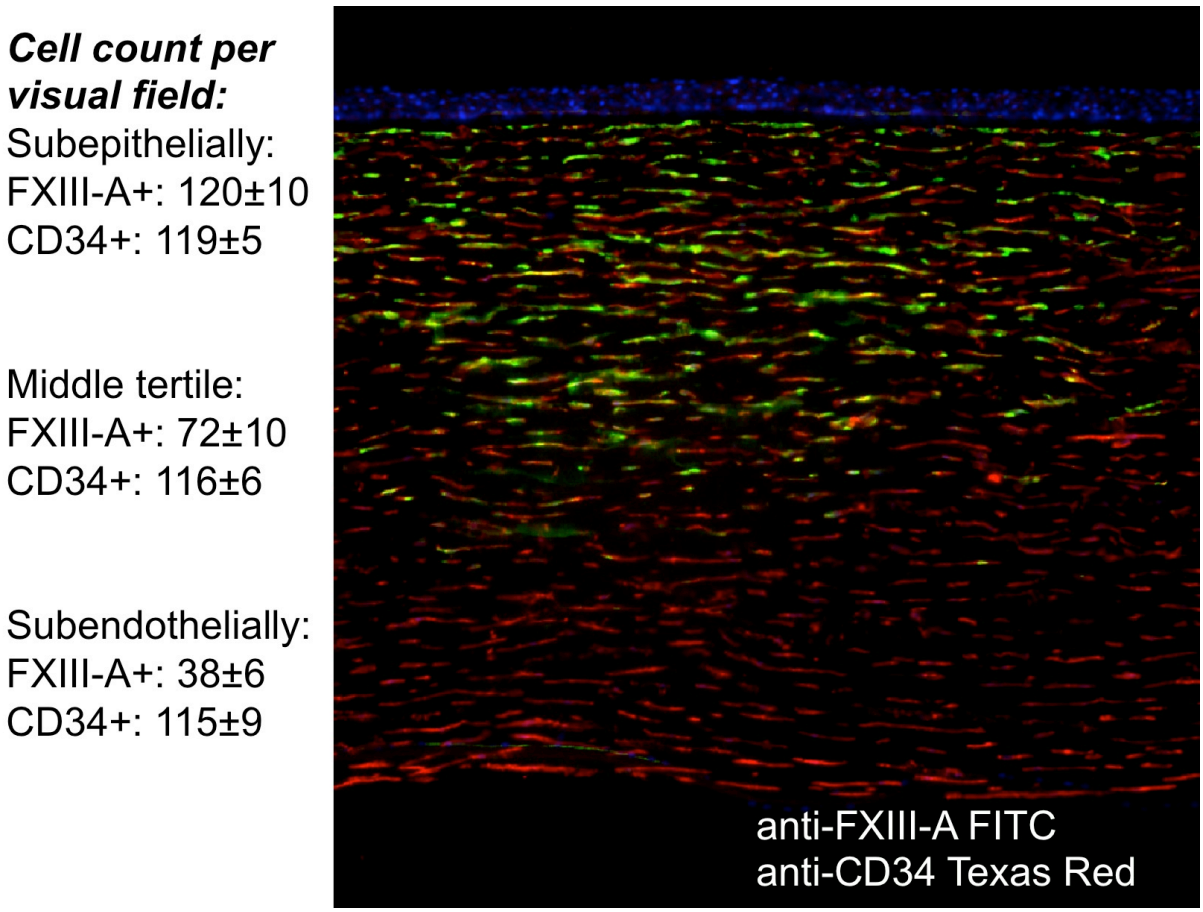

Figure 3. Co-localization of stromal keratocytes with anti-CD34 and anti-FXIII-A antibody. Uneven distribution of FXIII-A positive cells.

To continue the study on isolated cells we established the technique for isolation of keratocytes from cadaver human cornea using digestion of corneal tissue by collagenase. The cells were analyzed by flow cytometry. It was shown that over 90% of the isolated cells were intact keratocytes and 100% of them were CD34+. 68±13% of CD34+ keratocytes also contained FXIII-A. The subcellular distribution of FXIII-A in isolated keratocytes was studied on cytospin preparations by double immunofluorescent technique and laser scanning microscopy. As opposed to the membrane localization of CD34, FXIII-A was abundant in the cytoplasma (Figure 4); in a few cells it was also present in the nucleus.

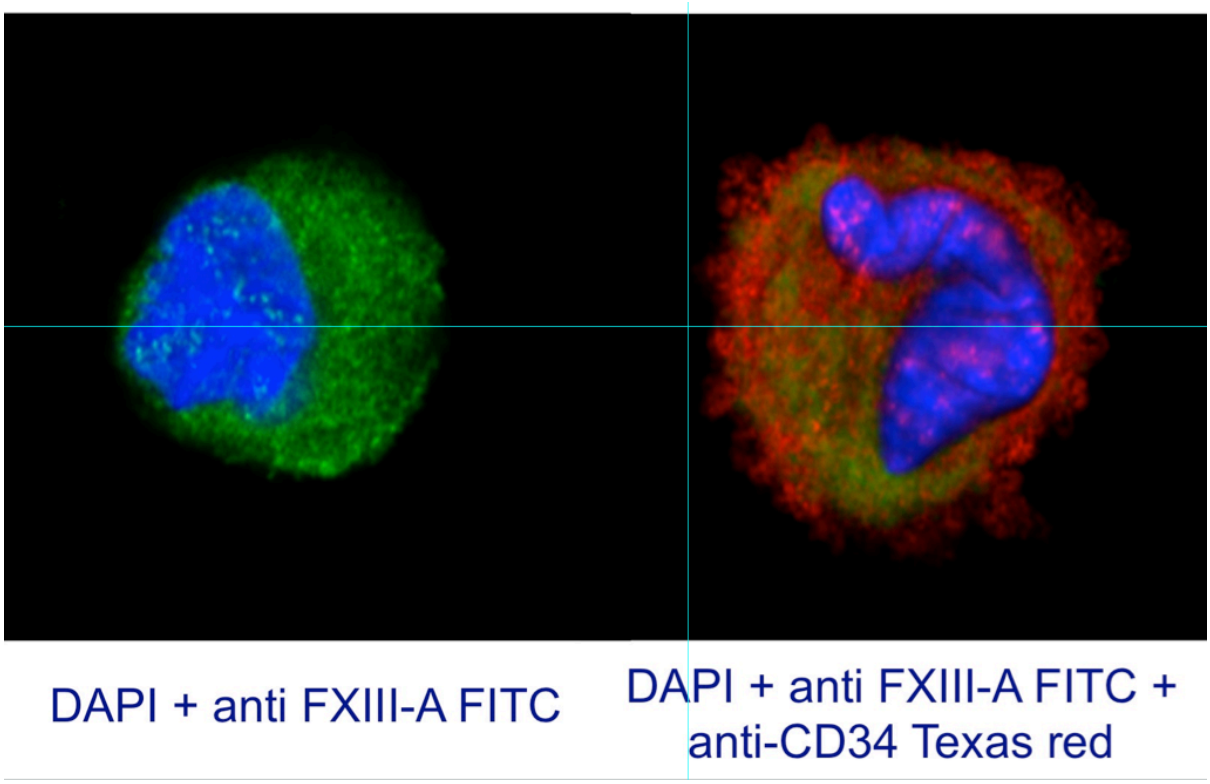

Figure 4. The distribution of FXIII-A and CD34 in isolated keratocytes

Although the discovery of cFXIII in the cornea warranted publication in itself we thought that the addition of data on the function of FXIII in the cornea would improve our chance to publish the findings in a high quality journal. First we demonstrated that cFXIII prepared from human platelets significantly accelerated the proliferation and migration of corneal epithelial cells. However this effect could not be repeated by recombinant cFXIII. The finding suggested to us that cFXIII within the cells is associated with so far unidentified growth factor(s), which might be responsible for the observed effect. The identity of these factors remains to be seen and it could be the target of a separate project.

Another approach was to examine the cornea of patients with FXIII-A deficiency. Although these patients are usually on prophylactic therapy with FXIII concentrate, FXIII from outside source does not penetrate the cornea and cannot substitute for the lack of cFXIII in the cornea. In Hungary we diagnosed two young adults with severe FXIII-A deficiency and characterized the deficiency. Cornea topography of those two patients revealed slight keratoconus (Figure 5). As FXIII-A deficiency is very rare, to collect at least 10 patients we organized an international cooperation with Finnish and Swiss scientists. We submitted the request for ethical approval to the ethical committees in Helsinki and Bern but to get the ethical approval took more time than we expected and that was one of the reasons why we asked for the extension of deadline for completing the project. Finally we were able to examine eight additional FXIII-A deficient patients (six Finnish and two Swiss patients) and in four of them the same topography was observed as in the Hungarian patients. Now we consider the study on FXIII-A in the cornea completed (the two Swiss patients could be examined only in December) and we are writing the manuscript.

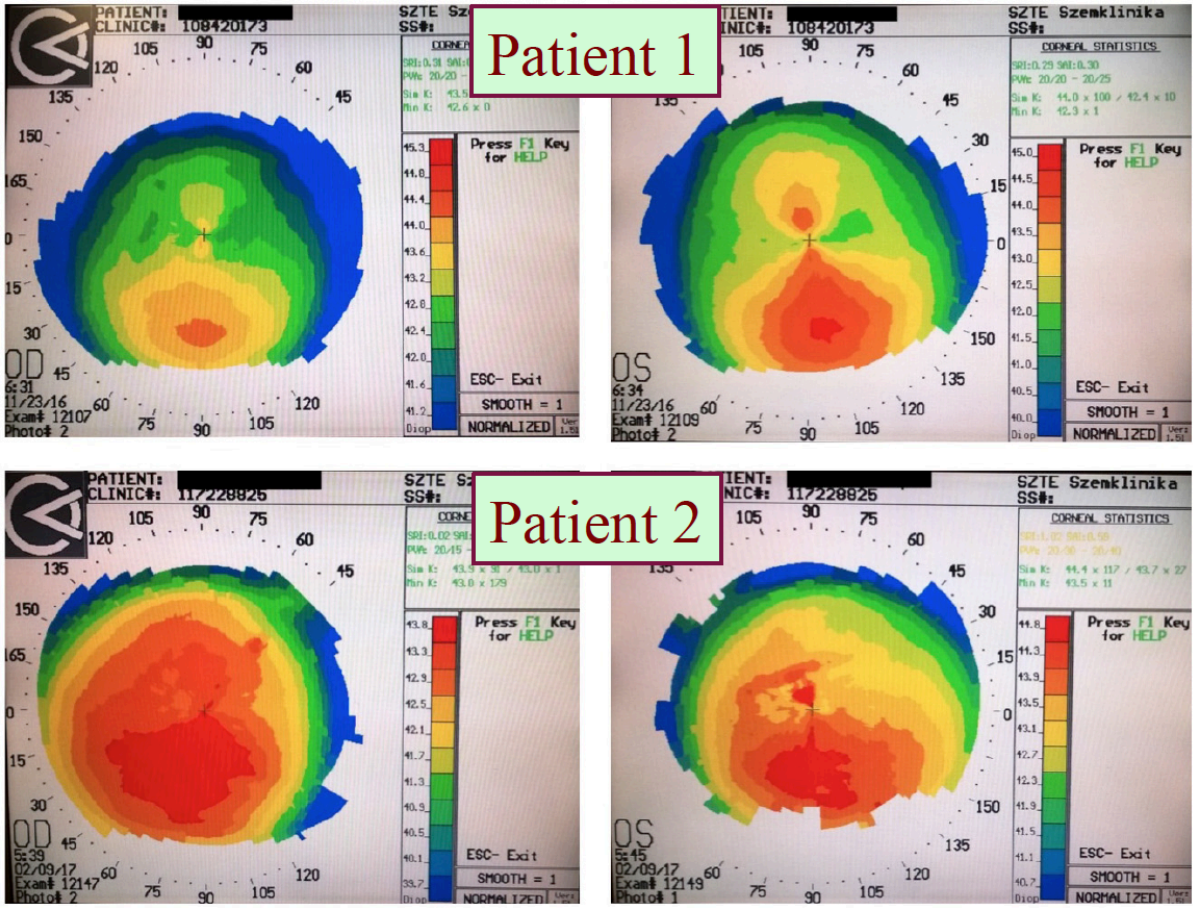

Figure 5. Cornea topography of two Hungarian FXIII-A deficient patients.

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Presentations on FXIII in the cornea:
Enzyme and Biocatalysis Congress, Seoul, South Korea, March 16-18, 2016 Muszbek L invited lecture “Transglutaminases in human cornea and lens”
2nd International Factor XIII Workshop September 25-October 2, 2016 Hévíz, Hungary (Muszbek et al. Abstract No.: T3/2 “Transglutaminases in human cornea and lens”, Orosz et al. abstract No T3/3 “Factor XIII in tears and its possible role in corneal wound healing”).
Gordon Research Conference on Towards Understanding and Modulating Transglutaminases in Human Diseases June 17-22, 2018, le Diablerets, France (Muszbek L invited lecture: Factor XIII in corneal keratocytes and its possible role in corneal matrix stabilization and wound healing.)
International Society on Thrombosis and Haemostasis, SSC July 18-21, 2018 Dublin, Ireland (Orosz et al. “The presence and function of cellular factor XIII in human cornea”).